AD_____

Award Number: DAMD17-98-1-8089

TITLE: Targeting of Oncogenic Proteins for Intracellular Degradation

PRINCIPAL INVESTIGATOR: Stephen Byers, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual (1 Jul 99 – 30 Jun 00)

4. TITLE AND SUBTITLE
Targeting of Oncogenic Proteins for Intracellular Degradation

5. FUNDING NUMBERS
DAMD17-98-1-8089

6. AUTHOR(S)
Stephen Byers, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

E-MAIL:
byerss@gunet.georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer. This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. We now propose an alternative approach in which an oncogenic protein is specifically targeted for intracellular degradation. In order to do this we will take advantage of the permeability properties of the third helix of the antennapedia protein. This will be used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way the ubiquitin-conjugating machinery will be selectively recruited to the target protein which should then be degraded by the proteosome. We will use the cytoplasmic signaling molecule β-catenin as a model system since its oncogenic activity is thought to be regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of β-catenin which increase its protein stability are oncogenic. The β-catenin binding peptide will be based on the region of the tumor suppressor protein APC which constitutively binds β-catenin.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
39

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

TABLE OF CONTENTS:

Introduction

Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer (1-5). This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. We now propose an alternative approach in which an oncogenic protein is specifically targeted for intracellular degradation. In order to do this we will take advantage of the permeability properties of the third helix of the antennapedia protein. This will be used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way the ubiquitin-conjugating machinery will be selectively recruited to the target protein, which should then be degraded by the proteosome. We will use the cytoplasmic signaling molecule β-catenin as one model system since its oncogenic activity is thought to be regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of β-catenin that increase its protein stability are oncogenic. The β-catenin binding peptide will be based on the region of the tumor suppressor protein APC that constitutively binds β-catenin. A second target will be ErbB-2 a tyrosine kinase strongly associated with breast cancer. The ErbB-2 binding peptide will consist either of the last three armadillo repeats of β-catenin known to constitutively bind ErbB-2 or the SH2 domain of grb2 which can only bind tyrosine phosphorylated ErbB-2. Generally, the work proposed is of great significance to the treatment of breast cancer. More specifically, proof of the principle that direct targeting of oncogenic proteins for intracellular degradation inactivates their transforming capabilities could lead to the development of novel therapuetic strategies based on this approach.

Body:

In the first annual report we described in detail the progress we had made in constructing targeting vectors for ErbB-2. Briefly the key research accomplishments of the first 12 months were:

1) A number of different targeting vectors have been constructed.

2) The targeting constructs have been transfected into three different cells of varying ErbB-2 status

3) Two of the constructs yield protein products of the predicted size indicating that the recombinant peptides are stable and can be expressed at relatively high levels.

4) The constructs were detected with an antibody directed at the FLAG tag indicating that it is accessible and does not interfere with protein production.

In the second year of funding we have concentrated on β-catenin. Since this grant was first submitted we have gathered important new information relating to the sites on β-catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we have found that IKKα, a kinase thought previously to only phosphorylate IκB proteins, is a potent and important kinase in the regulation of β-catenin phosphorylation and ubiquitination. In fact, our results point to IKKα rather than GSK-3β as the key enzyme in this process (see appendix 1). These results are extremely relevant to the present grant since they raise the possibility that sites other than, or in addition to, those we originally proposed to utilize in the construction of targeting vectors could be important. This work is summarized below and in a paper submitted for publication (appendix 1).

The IKK complex regulates β-catenin signaling:

The wnt/β-catenin and NFκB pathways regulate the transcription of genes that are involved in cell cycle control and cellular differentiation. In addition the NF-κB pathway is involved in the induction of the inflammatory response. β-catenin, a known oncogene, is an important component of the wnt signaling pathway and IκBα is an important regulator of the NF-κB pathway. Both proteins are phosphorylated at serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. The kinases that are important in the phosphorylation of these proteins have been intensely studied. The IKK complex is responsible for the phosphorylation of IκBα while GSK-3β is thought to regulate β-catenin phosphorylation. The IKK complex contains two kinases, IKKα and IKKβ. Gene disruption studies in mice indicate that IKKβ is the dominant component of the IKK complex involved in phosphorylation of IκBα. We now show that IKK also exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling in APC-mutant colon cancer cells with high endogenous β-catenin levels. However, only a dominant negative (DN) IKKα mutant increased β-catenin signaling and protein levels in cells with low endogenous β-catenin. In addition, DN IKKα, but not DN IKKβ, completely inhibited the ability of APC to decrease β-catenin signaling in colon cancer cells. These results indicate that, in contrast to IKK control of NFκB signaling, IKKα not IKKβ has the dominant role in the regulation of β-catenin activity.

Results and Discussion (refer to figures in appendix 1):

To determine whether IKK could regulate β-catenin signaling we first transfected SW480 colon cancer cells with the constitutively active IKK mutants and a reporter construct, which reflects β-catenin activity. These constitutively active mutants (CA IKKα and CA IKKβ (SS EE)) were generated by changing serine residues located within the highly conserved activation loop at position 176 and 180 in IKKα and 177 and 181 in IKKβ, which mimic the phosphorylation induced conformational change resulting in kinase activation. SW480 cells contain a truncated APC gene and as a result have very high β-catenin levels and signaling. To measure β-catenin signaling, we used a luciferase promoter containing Tcf/LEF response elements (TopFlash) and measured the lumens generated. A Tcf/LEF mutated luciferase reporter (FopFlash) was used as a control. β-catenin signaling decreased by 80-90% upon co-transfection of either CA IKKα or CA IKK β expression, (Figure 1A). In contrast, CA IKKα increased NF-κB reporter activity in the same experiment. To further investigate the requirement for IKK kinase activity, we next transfected kinase dead IKK α and β mutants, in which a conserved lysine in the ATP binding site at position 44 was mutated to either methionine in IKKα or alanine in IKKβ, into SW480 cells. Neither the kinase dead IKKα nor IKKβ mutant decreased β-catenin activity indicating that intact kinase function for both IKK α and β is necessary for their effects on β-catenin signaling (Figure 1B). These data indicate that IKKα and IKKβ are capable of regulating β-catenin signaling.

To more clearly address the role of these kinases, we transfected either dominant negative (DN) IKKα (S176/180A) or IKKβ (S177/181A) into SKBR3 breast cancer cells (Figure 1C). The alanine substitutions for serine residues in the activation loops of these kinases prevent kinase activation in response to TNFα and IL-1 stimulation. SKBR3 cells have very low levels of β-catenin and consequently the low levels of β-catenin activity permit easier detection of changes in its signaling activity. The DN IKKα increased β-catenin signaling over 5 fold and significantly increased β-catenin protein levels (Figure 1C). In contrast, the DN IKKβ had no effect on β-catenin signaling or protein levels but did inhibit NFκB activity. Neither CA IKKα nor CA IKKβ affected β-catenin signaling or protein levels in SKBR3 cells. These results indicate that blockade of endogenous IKKα activation but not of IKKβ activation has a direct effect on the activity of the IKK complex toward β-catenin in SKBR3 cells. This result contrasts with failure of DN

IKKα to block IKK activity toward IκBα. We next investigated the ability of DN IKKα to regulate the phosphorylation-dependent molecular weight shift observed in SKBR3 cells when β-catenin is over-expressed (Figure 1D). Consistent with a role for IKKα in regulating β-catenin phosphorylation the higher molecular weight form of β-catenin was absent in cells co-expressing DN IKKα. Western blot analysis was then performed to determine the levels of endogenous IKKα and β in the different cell lines used in these studies. We found SKBR3, SW480, and Cos-7 cells to have significantly more IKKα than IKKβ using the indicated IKKα and IKKβ antibodies while Jurkat cells, which were used as a positive control, expressed similar levels of IKKα and β (Figure 1E).

To determine whether IKKα influences the cellular localization of β-catenin, immunocytochemistry was performed using SKBR3 cells transfected with either HA-tagged DN IKKα or FLAG-tagged DN IKKβ. Cells were stained for either IKKα or IKKβ using antibodies directed against either the HA or FLAG tag as well as β-catenin. DN IKKα was found in both the nucleus and the cytoplasm in the majority of transfected cells (Figure 2 A1). β-catenin levels were significantly increased in DN IKKα transfected cells and in most cases β-catenin co-localized with DN IKKα in the nucleus (Figure 2 A2). In cells transfected with DN IKKβ, IKKβ was found only in the cytoplasm (Figure 2 B1) and there was no change in β-catenin levels or localization (Figure 2 B2). CA IKKα also localized to the nucleus (not shown). Western blot analysis of nuclear and cytoplasmic extracts confirmed the differential localization of DNIKKα and DNIKKβ in these cells.

To demonstrate whether IKK could regulate β-catenin activated transcription of a known target gene, a cyclin D1-luciferase promoter was transfected into SW480 cells. This cyclin D1 promoter construct (-163CD1Luc) contains Tcf/LEF sites as well as CREB, AP1, SP1, and NF-κB sites (31;37). As was the case with the TCF-reporter, TopFlash, both the constitutively active IKKα and IKKβ proteins decreased cyclin D1 promoter activity in SW480 cells (Figure 3). In contrast, a cyclin D1 promoter with mutated Tcf/LEF sites, but retaining the other regulatory elements, was not responsive to either IKKα or IKKβ. Because NFκB has been shown to positively regulate cyclin D1 promoter activity these data indicate that, in APC-mutant colon cancer cells, the activity of the -163 cyclin D1 promoter is predominantly regulated by IKK control of β-catenin not NF-κB signaling.

When wild type (WT) APC is over-expressed in SW480 cells, β-catenin signaling is dramatically reduced. In earlier studies, we found that GSK3β activity was not required for APC to decrease β-catenin signaling (7). To determine if IKK activity was required for APC to regulate β-catenin activity, SW480 cells were co-transfected with APC and DN IKKα or DN IKKβ (Figure 4). Remarkably, DN IKKα but not DN IKKβ completely inhibited the ability of APC to decrease β-catenin signaling. These results suggest that IKKα can directly alter β-catenin signaling perhaps by association with the IKK complex.

Finally, to determine whether IKKα could directly interact with β-catenin, immunoprecipitation was performed in Cos-7 kidney cells co-transfected with HA-tagged β-catenin and FLAG-tagged IKKα. Before immunoprecipitation cytoplasmic cell extracts were fractionated over a sizing column. Figure 5 shows that β-catenin is present in IKKα immunoprecipitates and that IKKα is also present in β-catenin immunoprecipitates. Thus IKKα can directly associate with a protein complex that includes β-catenin.

These data indicate that elevation of β-catenin protein levels, by wnt signaling for example, results in the increased activity of a kinase (IKKα?), which in turn phosphorylates the accumulated cytoplasmic and nuclear β-catenin to target it for degradation (Figure 6). In this model the IKK complex could be involved in the feedback regulation of pathways, such as wnt signaling, that result in elevated β-catenin levels. IKKα

protein levels are similar in cells that express very low levels of β-catenin (SKBR3 cells) and in cells that express extremely high levels of β-catenin (SW480) and indicates that β-catenin does not regulate IKKα protein levels. Consequently, we favor a model in which elevated β-catenin protein levels directly trigger activation of the IKK complex. Perhaps the role of APC is to facilitate the β-catenin-mediated activation of the IKK complex and complete the negative feedback loop. Our demonstration that constitutively active IKK can decrease β-catenin signaling independently of APC is consistent with this model. β-catenin can also regulate the levels of the ubiquitin ligase component βTRCP which can in turn increase IκBα degradation and subsequent NFκB activation as well as negatively regulating its own activity.

Taken together with the recent demonstration that GSK-3 is involved in the regulation of NFκB activity, these data indicate that there is a significant amount of "cross-talk" between these two pathways. Our results strongly suggest that substrate specificity of the IKK complex is regulated by the relative contribution of IKKα and IKKβ. Determining the precise mechanism by which IKK regulates β-catenin signaling as well as the factors specifying activity will further elucidate the relationship between these two important pathways.

Key Research Accomplishments:

1. Constitutively active IKKα and IKKβ can decrease β-catenin signaling
2. Dominant negative IKKα but not dominant negative IKKβ increases β-catenin signaling
3. Dominant negative IKKα but not dominant negative IKKβ reverse the effects of the tumor suppressor gene APC on β-catenin signaling
4. IKKα and β-catenin co-localize in the nucleus

Reportable outcomes:

The IKK complex regulates β-catenin signaling. Jarrett, Gaynor and Byers. Submitted 2000-Appendix 1

Conclusions:

Our discovery that IKKα is an important kinase in the normal regulation of β-catenin signaling is significant and has implications for the design of the β-catenin targeting vectors proposed in this grant. It is important that we elucidate the precise sites whereby IKKα regulates β-catenin phosphorylation because these sites are crucial to the design of the targeting vectors. In the next 12 months we will identify the IKKα phosphorylation sites on β-catenin and use this information to redesign the targeting vectors.

The IKK complex regulates β-catenin signaling

Christy Rothwell Jarrett*, Carmela Lamberti†, Keng-Mean Lin†, Stacey Kessler*, Richard B. Gaynor†, Stephen W. Byers*

*Department of Oncology, Lombardi Cancer Center and Department of Cell Biology, Georgetown University School of Medicine, Washington, DC 20007; †Division of Hematology-Oncology, Department of Medicine, Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Send correspondence to: Stephen Byers, PhD
Georgetown University Medical Center
3970 Reservoir Road, NW
Research Building E415
Washington, DC 20007
(202) 687-1813 (phone)
(202) 687-7505 (fax)
byerss@gunet.georgetown.edu

Running head: IKK regulates β-catenin
Key words: β-catenin, IKK, NF-κB, IκBα, wnt

ABSTRACT:

The wnt/β-catenin and IκB/NF-κB pathways are involved in cell cycle control, differentiation, and inflammation. IκB and β-catenin are phosphorylated at similar consensus serines and targeted for ubiquitination by the same ubiquitin ligase complex. The IKK complex that phosphorylates IκB contains two kinases, IKKα and IKKβ. We show that IKK also exists in a complex with β-catenin and that both IKKα and IKKβ can regulate signaling activity. However, only dominant negative IKKα increased endogenous β-catenin signaling and protein levels. IKKα was also required for the tumor suppressor APC to regulate β-catenin signaling. In contrast to IKK control of NF-κB signaling, IKKα not IKKβ has the dominant role in the regulation of β-catenin activity.

INTRODUCTION:

β-catenin is an oncogene involved in signaling by the wnt pathway. Upon wnt signaling, β-catenin accumulates in the cytoplasm and nucleus where it interacts with Tcf/LEF transcription factors. This complex activates transcription of several genes, including cyclin D1 and c-myc, indicating that this pathway may be involved in cell cycle regulation (He et al., 1998; Korinek et al., 1997; Orford et al., 1999; Shtutman et al., 1999; Tetsu O, 1999). In the absence of wnt signaling, cytoplasmic β-catenin associates with a complex containing the tumor supressor gene adenomatous polyposis coli (APC), axin, and GSK3β (Munemitsu et al., 1995; Munemitsu et al., 1996; Orford et al., 1997; Polakis, 1997). The activity of this complex results in phosphorylation of β-catenin at N-terminal serine and threonine residues. Mutations in this N-terminal region or mutations of the APC gene lead to accumulation of β-catenin and are associated with many different cancers, including melanoma and colon cancer (reviewed in (Morin, 1999)). Once phosphorylated β-catenin is recognized by F box/WD 40 repeat containing proteins, targeting it for ubiquitination by the SCF E3 ubiquitin ligase and subsequent proteasomal degradation (Hart et al., 1999; Kitagawa et al., 1999; Winston et al., 1999; Fuchs et al., 1999). Although GSK-3β is clearly involved in the wnt-mediated regulation of β-catenin stability, several studies indicate that other kinases may also be involved in regulation of β-catenin signaling. For example, both typical and atypical PKCs have been implicated in β-catenin regulation (Cook et al., 1996; Orford et al., 1997). However, LiCl, an inhibitor of GSK3β, does not significantly alter the ability of APC to down-regulate β-catenin activity (Easwaran et al., 1999a). In addition, the GSK3β knockout mouse does not exhibit any changes in β-catenin levels and is morphologically

normal up to 12 days of gestation (Hoeflich et al., 2000). Remarkably, GSK3β knockout mice are characterized by defects similar to those of mice lacking components of the NF-κB pathway.

There are several other connections between β-catenin regulation and regulation of IκBα, which inhibits NF-κB by sequestering it in the cytoplasm. The IκBα kinase complex, IKK, regulates NF-κB activation by phosphorylating its inhibitors IκBα, IκBβ, and IκBε on two closely spaced serine residues and targeting them for ubiquitination using the same F box/SCF machinery used for ubiquitination of β-catenin (Fuchs et al., 1999; Winston et al., 1999; Mercurio et al., 1997; Li et al., 1998; Regnier et al., 1997). IKK recognizes and phosphorylates IκBα at a DSGIHS consensus sequence in the N-terminal region including serines 32 and 36. This consensus sequence is also found in the N-terminal of β-catenin at serines 33 and 37 (Orford et al., 1997).

The IKK complex is composed of a number of different proteins including the active catalytic components IKKα and IKKβ and the scaffold protein IKKγ/NEMO (Rothwarf et al., 1998; Yamaoka et al., 1998; Karin, 1999a). Several different stimuli including tumor necrosis factor α (TNFα) and interleukin 1 (IL-1) can stimulate the activity of the IKK complex. A number of kinases including MEKK1, NF-κB inducing kinase (NIK), and atypical PKCs can activate IKK (Lee et al., 1998; Ling et al., 1998; Nakano et al., 1998; Ninomiya-Tsuji et al., 1999). Mechanical stress acting through integrins can also activate IKK (Bhullar et al., 1998). Once activated, the IKK complex phosphorylates IκB proteins, which lead to their degradation resulting in the release of the NF-κB proteins and their migration to the nucleus where they activate transcription of certain cellular genes. The NF-κB proteins are known to play a role in

inflammatory responses as well as in apoptosis, cell survival, and development (reviewed in (Karin and Delhase, 2000)).

IKKβ has higher activity for the IκB proteins and has a more significant role in the NF-κB pathway in response to activation such as TNFα and IL-1 than does IKKα (Delhase et al., 1999; Li et al., 1999b; Li et al., 1999c). For example, IKKβ knockout mice die around embryonic day 12.5 from severe liver apoptosis, a phenotype that is similar to knockouts of the NF-κB component p65 and of GSK3β. Fibroblasts taken from these embryos demonstrate decreased NF-κB activity and increased apoptosis due to unopposed TNFα stimulation. IKKα cannot compensate for the loss of IKKβ indicating that IKKβ is absolutely necessary for the regulation of this pathway. Knockouts of the IKKγ/NEMO gene which is critical for the control of IKKβ activity also result in severe hepatic apoptosis and defects in NF-κB activation in response to TNFα and IL-1 treatment (Rudolph et al., 2000). In contrast, IKKα knockouts die perinatally with skeletal abnormalities including fused vertebrae, syndactyly, and missing phalanges (Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999). Upon TNFα and IL-1 stimulation, IKKα-/- cells exhibit somewhat reduced IκBα degradation and NF-κB activity but the defect is not as severe as seen with disruption of the IKKβ gene. Moreover, these mice do not exhibit the severe hepatic apoptosis that characterizes the IKKβ, IKKγ/NEMO, and GSK-3β knockout mice. These results demonstrate different roles for IKKα and IKKβ. In addition, IKKα knockout mice exhibit an unusual 10-fold thickening of the epidermis due to hyperproliferation of basal keratinocyte stem cells. Significantly, increased β-catenin levels are also associated with uncontrolled proliferation of epidermal basal cells and there are examples of

wnt and LEF-1 involvement in hair and epidermal differentiation (Zhu and Watt, 1999; Gat et al., 1998).

In the present study we investigated the ability of IKK to regulate β-catenin signaling activity. We found that IKK exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling. However, only a dominant negative (DN) IKKα mutant increased β-catenin signaling as well as protein levels. In addition, DN IKKα, but not DN IKKβ, inhibited the ability of APC to decrease β-catenin signaling. These results suggest that the IKK proteins are involved in β-catenin regulation.

Results:

To determine whether IKK could regulate β-catenin signaling we first transfected SW480 colon cancer cells with the constitutively active IKK mutants and a reporter construct, which reflects β-catenin activity. These constitutively active mutants (CA IKKα and CA IKKβ (SS→EE)) were generated by changing serine residues located within the highly conserved activation loop at position 176 and 180 in IKKα and 177 and 181 in IKKβ, which mimic the phosphorylation induced conformational change resulting in kinase activation. SW480 cells contain a truncated APC gene and as a result have very high β-catenin levels and signaling. To measure β-catenin signaling, we used a luciferase promoter containing Tcf/LEF response elements (TopFlash) and measured the lumens generated. A Tcf/LEF mutated luciferase reporter (FopFlash) was used as a control. β-catenin signaling decreased by 80-90% upon co-transfection of either CA IKKα or CA IKK β expression, (Figure 1A). In contrast, CA IKKα increased NF-κB reporter activity in the same experiment. To further investigate the requirement for IKK kinase activity, we next transfected kinase dead IKK α and β mutants, in which a conserved lysine in the ATP binding site at position 44 was mutated to either methionine in IKKα or alanine in IKKβ, into SW480 cells. Neither the kinase dead IKKα nor IKKβ mutant decreased β-catenin activity indicating that intact kinase function for both IKK α and β is necessary for their effects on β-catenin signaling (Figure 1B). These data indicate that IKKα and IKKβ are capable of regulating β-catenin signaling.

To more clearly address the role of these kinases, we transfected either dominant negative (DN) IKKα (S176/180A) or IKKβ (S177/181A) into SKBR3 breast cancer cells (Figure 1C).

The alanine substitutions for serine residues in the activation loops of these kinases prevent kinase activation in response to TNFα and IL-1 stimulation. SKBR3 cells have very low levels of β-catenin and consequently the low levels of β-catenin activity permit easier detection of changes in its signaling activity. DN IKKα increased β-catenin signaling over 5 fold and significantly increased β-catenin protein levels (Figure 1C). In contrast, DN IKKβ had no effect on β-catenin signaling or protein levels. Neither CA IKKα nor CA IKKβ affected β-catenin signaling or protein levels in SKBR3 cells. These results indicate that blockade of endogenous IKKα activation but not of IKKβ activation has a direct effect on the activity of the IKK complex toward β-catenin in SKBR3 cells. This result contrasts with the failure of IKKα inhibition to disrupt IKK activity toward IκBα (Karin and Delhase, 2000). We next investigated the ability of DN IKKα to regulate the phosphorylation-dependent molecular weight shift observed in SKBR3 cells when β-catenin is over-expressed (Figure 1D). Consistent with a role for IKKα in regulating β-catenin phosphorylation the higher molecular weight form of β-catenin was absent in cells co-expressing DN IKKα. Western blot analysis was then performed to determine the levels of endogenous IKKα and β in the different cell lines used in these studies. We found SKBR3, SW480, and Cos-7 cells to have significantly more IKKα than IKKβ using the indicated IKKα and IKKβ antibodies while Jurkat cells, which were used as a positive control, expressed similar levels of IKKα and β (Figure 1E).

To determine whether IKKα influences the cellular localization of β-catenin, immunocytochemistry was performed using SKBR3 cells transfected with either HA-tagged DN IKKα or FLAG-tagged DN IKKβ. Cells were stained for either IKKα or IKKβ using antibodies

directed against either the HA or FLAG tag as well as β-catenin. DN IKKα was found in both the nucleus and the cytoplasm in the majority of transfected cells (Figure 2 A1). β-catenin levels were significantly increased in DN IKKα transfected cells and in most cases β-catenin co-localized with DN IKKα in the nucleus (Figure 2 A2). In cells transfected with DN IKKβ, IKKβ was found only in the cytoplasm (Figure 2 B1) and there was no change in β-catenin levels or localization (Figure 2 B2). CA IKKα also localized to the nucleus (not shown). Western blot analysis of nuclear and cytoplasmic extracts confirmed the differential localization of IKKα and IKKβ in these cells (Figure 2C).

To demonstrate that IKK could regulate β-catenin activated transcription of a known target gene, a cyclin D1-luciferase promoter was transfected into SW480 cells. This cyclin D1 promoter construct (-163CD1Luc) contains Tcf/LEF sites as well as CREB, AP1, SP1, and NF-κB sites (Pestell et al., 1999; Shtutman et al., 1999). As was the case with the TCF-reporter, TopFlash, both the constitutively active IKKα and IKKβ proteins decreased cyclin D1 promoter activity in SW480 cells (Figure 3). In contrast, a cyclin D1 promoter with mutated Tcf/LEF sites, but retaining the other regulatory elements, was not responsive to either IKKα or IKKβ. Because NF-κB has been shown to positively regulate cyclin D1 promoter activity these data indicate that, in APC-mutant colon cancer cells, the activity of the -163 cyclin D1 promoter is predominantly regulated by IKK control of β-catenin not NF-κB signaling (Shtutman et al., 1999).

When wild type (WT) APC is over-expressed in SW480 cells, β-catenin signaling is dramatically reduced. In earlier studies, we found that GSK3β activity was not required for APC to decrease β-catenin signaling (Easwaran et al., 1999a). To determine if IKK activity was required for APC to regulate β-catenin activity, SW480 cells were co-transfected with APC and DN IKKα or DN IKKβ (Figure 4). Remarkably, DN IKKα but not DN IKKβ completely inhibited the ability of APC to decrease β-catenin signaling. These results suggest that IKKα can directly alter β-catenin signaling perhaps by association with the IKK complex.

Finally, to determine whether IKKα could directly interact with β-catenin, immunoprecipitation was performed in Cos-7 kidney cells co-transfected with HA-tagged β-catenin and FLAG-tagged IKKα. Before immunoprecipitation cytoplasmic cell extracts were fractionated over a sizing column. Figure 5 shows that β-catenin is present in IKKα immunoprecipitates and that IKKα is also present in β-catenin immunoprecipitates. Thus IKKα can directly associate with a protein complex which includes β-catenin.

Discussion:

Several studies point to separate roles for IKKα and IKKβ in regulating the activity of the IKK complex (reviewed in (Karin, 1999b)). IKKβ activity is essential for the ability of the IKK complex to regulate IκB protein stability and thus control the transcription of NF-κB regulated genes. Although both exogenously expressed and endogenous IKKα can regulate NF-κB activity, it does not appear to have a dominant role in activating the NF-κB pathway (Li et al., 1999a). These results suggest that IKKα likely has targets other than the IκB proteins. In the present study we show that the IKK complex can also regulate β-catenin protein levels and signaling activity. This is the first demonstration of an IKK substrate other than the IκB proteins.

In contrast to the dominant role of IKKβ in NF-κB signaling, our experiments show that IKKα is the dominant IKK component involved in β-catenin regulation. When over-expressed, both constitutively active IKKα and IKKβ can decrease β-catenin signaling in SW480 cells, which have high endogenous levels of β-catenin. However, these kinases have no detectable effect in SKBR3 cells, which have low endogenous levels of β-catenin. Only the dominant negative IKKα, but not IKKβ, can increase β-catenin protein levels and signaling in SKBR3 cells. Furthermore, only the dominant negative IKKα, but not IKKβ, can reverse the inhibitory effects of APC on β-catenin signaling in SW480 cells. Taken together these data suggest a model in which signals that activate the IKK complex through IKKα can target a complex containing β-catenin whereas signals that activate the IKK complex through IKKβ target it for

IκBα. Alternatively, it is possible that a subcomplex of IKKα homodimers can bind to β-catenin. A role for IKKα in the regulation of β-catenin signaling in epithelial cells is consistent with the phenotype of the IKKα -/- mouse and of epidermal cells over-expressing β-catenin. Each of these phenotypes is characterized by hyperproliferation of basal stem cells (Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999; Zhu and Watt, 1999). A role for the IKKα/β-catenin/TCF pathway in the regulation of epithelial stem cell proliferation is also consistent with the phenotype of the TCF4 -/- mouse which completely lacks the stem cell compartment of the intestine (Parvinen et al., 1986).

The precise mechanism by which IKK regulates β-catenin is not clear but is likely to involve phosphorylation of one or more of the N-terminal serine and threonine residues known to regulate the stability and perhaps nuclear localization of both proteins (Winston et al., 1999; Fuchs et al., 1999). Like β-catenin, IκBα has a function in both the cytoplasm and the nucleus (Arenzana-Seisdedos et al., 1997). In addition to its role as a cytoplasmic inhibitor of NF-κB nuclear translocation, nuclear IκBα can also directly inhibit NF-κB mediated transcriptional activation by inhibiting its DNA binding properties. Similarly, cytoplasmic β-catenin is involved in cell-cell adhesion and in the indirect regulation of PKA-regulated genes in addition to its role as a nuclear co-activator of TCF/LEF regulated transcription (Xu et al., 2000). Remarkably, both IκB and β-catenin can also interact with and regulate the function of retinoid receptors (Easwaran et al., 1999b; Na et al., 1998). IκB can negatively regulate RXR-mediated transcription whereas β-catenin can act as a co-activator of RAR-mediated transcription. Our results show that both IKKα and IKKβ are present in the cytoplasm but that only IKKα is able to localize to the nucleus. This differential localization of the two IKK kinases may be relevant for

the precise regulation of both nuclear and cytoplasmic functions of IκBα and β-catenin. For example it is possible that IKKα could have two roles in the regulation of β-catenin signaling. IKKα could phosphorylate β-catenin and target it for ubiquitination and could also directly interact with it to prevent its co-activator function in the nucleus. Perhaps IKKβ can also regulate β-catenin phosphorylation in the cytoplasm but lacks the nuclear activity of IKKα.

Although APC is most commonly associated with wnt signaling, genetic and biochemical studies differ on its role in this pathway; in some systems it antagonizes wnt signaling and in others it potentiates it (reviewed in (Seidensticker and Behrens, 2000)). Similarly, although genetic studies show that GSK-3β is a component of the wnt pathway and that inhibition of GSK-3β activity elevates β-catenin signaling and proteins levels in several different in vitro systems, GSK-3β activity is not required for APC to regulate β-catenin signaling or protein levels (Easwaran et al., 1999a). However the present study shows that IKKα (but not IKKβ) is required for APC-mediated inhibition of β-catenin signaling. Other data shows that the phosphorylation event that results in the formation of the slower migrating form of β-catenin only occurs when β-catenin is overexpressed in cells expressing wild-type APC (Fig. 1D). The generation of this slower migrating species is completely blocked by co-expression of DN IKKα. These data indicate that elevation of β-catenin protein levels, by wnt signaling for example, results in the increased activity of a kinase (IKKα?), which in turn phosphorylates the accumulated cytoplasmic and nuclear β-catenin to target it for degradation (Figure 6). In this model the IKK complex could be involved in the feedback regulation of pathways, such as wnt signaling, that result in elevated β-catenin levels. IKKα protein levels are similar in cells that

express very low levels of β-catenin (SKBR3 cells) and in cells that express extremely high levels of β-catenin (SW480) and indicates that β-catenin does not regulate IKKα protein levels. Consequently, we favor a model in which elevated β-catenin protein levels directly trigger activation of the IKK complex. Perhaps the role of APC is to facilitate the β-catenin-mediated activation of the IKK complex and complete the negative feedback loop (Figure 6). Our demonstration that constitutively active IKK can decrease β-catenin signaling independently of APC is consistent with this model. β-catenin can also regulate the levels of the ubiquitin ligase component βTRCP which can in turn increase IκBα degradation and subsequent NF-κB activation as well as negatively regulating its own activity (Spiegelman et al., 2000). Wnt signaling has also been reported to activate NF-κB reporters (Bournat et al., 2000). Taken together with the recent demonstration that GSK-3 is involved in the regulation of NF-κB activity, these data indicate that there is a significant amount of "cross-talk" between these two pathways (Hoeflich et al., 2000; Bournat et al., 2000). Our results strongly suggest that substrate specificity of the IKK complex is regulated by the relative contribution of IKKα and IKKβ. Determining the precise mechanism by which IKK regulates β-catenin signaling as well as the factors specifying activity will further elucidate the relationship between these two important pathways.

Experimental Procedures:

Cell culture and Transfections: SW480 colon cancer cells, SKBR3 breast cancer cells, and Cos-7 kidney cells were grown in DMEM supplemented with 5% fetal bovine serum. SW480 cells were transfected using lipofectamine plus (GibcoBRL). SKBR3 and Cos-7 cells were transfected using calcium phosphate (Promega). F. Mercurio kindly provided the IKKα (S176/180E and A) and IKK β (S177/181E and A) mutants (Mercurio et al., 1997). B. Vogelstein kindly provided APC. R. Pestell kindly provided the cyclin D1-luciferase promoter. Tularik Inc kindly provided the IKKα (K44M) and IKKβ (K44A) mutants (Woronicz et al., 1997).

Tcf/LEF Reporter Assay: Cells were plated at ~100,000 cells/well in a 12 well plate. After 24 hours, cells were transfected with the indicated DNA as well as TopFlash, which is a luciferase reporter containing Tcf/LEF response elements, or FopFlash, which is a Tcf/LEF mutated luciferase reporter, (described in (van de Wetering et al., 1997)) and Renilla luciferase. All transfections were done in triplicate and repeated at least three times with the Tcf/LEF reporter activity measured in lumens after 48 hours using the luciferase assay (Promega). Dose responses were also performed and optimal doses chosen for these experiments.

Western Blotting: Cells were grown to confluence in 100 mm dishes (48 hours after transfection), washed twice with phosphate buffered saline (PBS), and lysed for 10 minutes on ice with NP-40 lysis buffer containing 1% NP-40 and protease inhibitors (1mM sodium vanadate, 50 mM sodium fluoride, and Boehringer Mannheim complete mini EDTA-free protease inhibitor cocktail). Lysates were centrifuged at 12,000 rpm at 4°C for 10 minutes.

Protein content was measured by the BCA protein assay (Pierce). Western blotting was performed as previously described using β-catenin monoclonal antibody (Transduction Laboratories) at a 1:1000 dilution in 5% milk (Sommers et al., 1994). Monoclonal HA antibody (Boerhinger Mannheim) was used at a concentration of 1 μg/ml, monoclonal FLAG antibody (Eastman Kodak) was used at a 1:500 dilution, and myc antibody was used at a concentration of 1 μg/ml. IKKα and IKKβ monoclonal antibodies (Upstate Biotechnology) were used at a concentration of 0.5 μg/ml. The blots were developed using chemiluminescent detection (ECL-Amersham Pharmacia Biotech).

Immunocytochemistry: Cells were plated on 18mm coverslips in 12 well plates at ~ 50,000 cells/well. Cells were transfected as above and fixed 48 hours later in 2% paraformaldehyde and permeabilized with 0.2% Triton. Monoclonal HA antibody (Babco) was used at a 1:1000 dilution and monoclonal FLAG antibody was used at a 1:500 dilution. Polyclonal β-catenin antibody (kindly provided by David Rimm) was used at a 1:2000 dilution. All primary antibodies were incubated for 1 hour at room temperature. Fluorescein and Texas Red conjugated secondary antibodies (Kirkegaard and Perry Laboratories) were used at a 1:100 dilution and incubated for 1 hour at room temperature. Imaging was done on an Olympus Fluoview Confocal Laser Scanning Microscope.

Fractionation of Cellular Extracts: Cytoplasmic extracts were prepared according to Li et al (Li et al., 1999d) with slight modifications. Cos-7 cells were plated in 100 mM dishes (10^7 cells) and co-transfected with Flag-tagged IKKα and HA-tagged β-catenin. Prior to harvest, cells were washed twice with cold phosphate-buffered saline (PBS). Cells were next resuspended in buffer

A (10 mM Hepes [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β-glycerolphosphate, 0.5 μM okadaic acid, 1 mM sodium orthovanadate) and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, cells were lysed in Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH3.7 rotor). The supernatants were collected and mixed with 0.11 volume of buffer B (0.3 M Hepes [pH 7.9], 30 mM $MgCl_2$, and 1.4 M NaCl) and then centrifuged at 100,000 xg for 60 min at 4°C. The supernatants after ultra-centrifugation were termed S100. The remaining pellets were incubated with 3 volumes of buffer C (20 mM Hepes [pH 7.9], 1.5 mM MgCl2, 0.42 M NaCl, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM EDTA, supplemented with phosphatase and protease inhibitors as described above) for 30 min at 4 °C. After centrifugation at 14,000 x g on a microfuge for 30 min at 4°C, the supernatants were collected and termed nuclear extracts.

Gel Filtration Chromatography: S100 fractions were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with buffer D (20 mM Hepes [pH 7.9], 0.1 M KCl, 0.5 mM PMSF, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol). Protein markers (Sigma) used for the calibration of the column include bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), b-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa).

Protein Association Studies and Western Blot Analysis: For protein association studies, equal volumes from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1 μg of indicated antibodies or normal mouse IgG. Immune complexes were collected with protein G-agarose (Sigma) for 2-3 h at 4 °C. Immunoprecipitates were analyzed by Western blotting using a chemiluminiscence system (ECL-Amersham Pharmacia Biotech).

Acknowledgements

This work was supported by DOD grants DAMD 17-99-1-9197, DAMD 17-98-1-8089, and a grant from the Komen Foundation to C.R.J. and S.W.B. as well as by NIH grant CA74128 to R.G.B. We thank Lindsey Murray for her technical support. We also acknowledge the support of the Lombardi Cancer Center SPORE Imaging and Microscopy Core Facility.

Reference List

Arenzana-Seisdedos,F., Turpin,P., Rodriguez,M., Thomas,D., Hay,R.T., Virelizier,J.L., and Dargemont,C. (1997). Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm. J Cell Sci. *110*, 369-378.

Bhullar,I.S., Li,Y.S., Miao,H., Zandi,E., Kim,M., Shyy,J.Y., and Chien,S. (1998). Fluid shear stress activation of IkappaB kinase is integrin-dependent. J Biol Chem. *273*, 30544-30549.

Bournat,J.C., Brown,A.M., and Soler,A.P. (2000). Wnt-1 dependent activation of the survival factor NF-kappaB in PC12 cells. J Neurosci. Res *61*, 21-32.

Cook,D., Fry,M.J., Hughes,K., Sumathipala,R., Woodgett,J.R., and Dale,T.C. (1996). Wingless inactivates glycogen synthase kinase-3 via an intracellular signaling pathway which involves a protein kinase C. EMBO. J. *15*, 4526-4536.

Delhase,M., Hayakawa,M., Chen,Y., and Karin,M. (1999). Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science *284*, 309-313.

Easwaran,V., Pishvaian,M., Salimuddin, and Byers,S. (1999b). Cross regulation of beta catenin/LEF/TCF and retinoid signaling pathways. Current Biology *9*, 1415-1418.

Easwaran,V., Song,V., Polakis,P., and Byers,S. (1999a). The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin- lymphocyte enhancer-binding factor signaling. J. Biol. Chem. *274*, 16641-16645.

Fuchs,S.Y., Chen,A., Xiong,Y., Pan,Z.Q., and Ronai,Z. (1999). HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. Oncogene *18*, 2039-2046.

Gat,U., DasGupta,R., Degenstein,L., and Fuchs,E. (1998). De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell *95*, 605-614.

Hart,M., Concordet,J.P., Lassot,I., Albert,I., del los,S.R., Durand,H., Perret,C., Rubinfeld,B., Margottin,F., Benarous,R., and Polakis,P. (1999). The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr. Biol *9*, 207-210.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-Myc as a target of the APC pathway. Science *281*,1509-1512.

Hoeflich,K.P., Luo,J., Rubie,E.A., Tsao,M.S., Jin,O., and Woodgett,J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86-90.

Hu,Y., Baud,V., Delhase,M., Zhang,P., Deerinck,T., Ellisman,M., Johnson,R., and Karin,M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science *284*, 316-320.

Karin,M. (1999a). How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene *18*, 6867-6874.

Karin,M. (1999b). The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. J Biol Chem. *274*, 27339-27342.

Karin,M. and Delhase,M. (2000). The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin. Immunol. *12*, 85-98.

Kitagawa,M., Hatakeyama,S., Shirane,M., Matsumoto,M., Ishida,N., Hattori,K., Nakamichi,I., Kikuchi,A., and Nakayama,K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. EMBO J. *18*, 2401-2410.

Korinek,V., Barker,N., Morin,P.J., van Wichen,D., de Weger,R., Kinzler,K.W., Vogelstein,B., and Clevers,H. (1997). Constitutive transcriptional activation by a b-catenin-Tcf Complex in $APC^{-/-}$ colon carcinoma. Science *275*, 1784-1787.

Lee,F.S., Peters,R.T., Dang,L.C., and Maniatis,T. (1998). MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. Proc. Natl. Acad. Sci. U. S. A. *95*, 9319-9324.

Li,J., Peet,G.W., Pullen,S.S., Schembri-King,J., Warren,T.C., Marcu,K.B., Kehry,M.R., Barton,R., and Jakes,S. (1998). Recombinant IkappaB kinases alpha and beta are direct kinases of Ikappa Balpha. J. Biol Chem. *273*, 30736-30741.

Li,Q., Lu,Q., Hwang,J.Y., Buscher,D., Lee,K.F., Izpisua-Belmonte,J.C., and Verma,I.M. (1999a). IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev. *13*, 1322-1328.

Li,Q., Van,A.D., Mercurio,F., Lee,K.F., and Verma,I.M. (1999c). Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science *284*, 321-325.

Li,X.H., Murphy,K.M., Palka,K.T., Surabhi,R.M., and Gaynor,R.B. (1999d). The human T-cell leukemia virus type-1 Tax protein regulates the activity of the IkappaB kinase complex. J Biol Chem. *274*, 34417-34424.

Li,Z.W., Chu,W., Hu,Y., Delhase,M., Deerinck,T., Ellisman,M., Johnson,R., and Karin,M. (1999b). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J. Exp. Med. *189*, 1839-1845.

Ling,L., Cao,Z., and Goeddel,D.V. (1998). NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc. Natl. Acad. Sci. U. S. A. *95*, 3792-3797.

Mercurio,F., Zhu,H., Murray,B.W., Shevchenko,A., Bennett,B.L., Li,J., Young,D.B., Barbosa,M., Mann,M., Manning,A., and Rao,A. (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF- kappaB activation. Science *278*, 860-866.

Morin,P.J. (1999). beta-catenin signaling and cancer. Bioessays *21*, 1021-1030.

Munemitsu,S., Albert,I., Rubinfeld,B., and Polakis,P. (1996). Deletion of an amino-terminal sequence stabilizes b-catenin in vivo and promotes hyperphosphorylation of the adenomatous polyposis coli tumor suppressor protein. Mol. Cell Biol. *16*, 4088-4094.

Munemitsu,S., Albert,I., Souza,B., Rubinfeld,B., and Polakis,P. (1995). Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. U. S. A. *92*, 3046-3050.

Na,S.Y., Kim,H.J., Lee,S.K., Choi,H.S., Na,D.S., Lee,M.O., Chung,M., Moore,D.D., and Lee,J.W. (1998). IkappaBbeta interacts with the retinoid X receptor and inhibits retinoid-dependent transactivation in lipopolysaccharide-treated cells. J. Biol. Chem. *273*, 3212-3215.

Nakano,H., Shindo,M., Sakon,S., Nishinaka,S., Mihara,M., Yagita,H., and Okumura,K. (1998). Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc. Natl. Acad. Sci. U. S. A. *95*, 3537-3542.

Ninomiya-Tsuji,J., Kishimoto,K., Hiyama,A., Inoue,J., Cao,Z., and Matsumoto,K. (1999). The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature *398*, 252-256.

Orford,K., Crockett,C., Jensen,J.P., Weissman,A.M., and Byers,S.W. (1997). Serine phosphorylation-regulated ubiquitination and degradation of beta catenin. JBC *272*, 24735-24738.

Orford,K., Orford,C.C., and Byers,S.W. (1999). Exogenous expression of beta catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest. J. Cell Biol *146*, 1-13.

Parvinen,M., Vihko,K.K., and Toppari,J. (1986). Cell interactions during the seminiferous epithelial cycle. Int. Rev. Cytol. *104*, 115-151.

Pestell,R.G., Albanese,C., Reutens,A.T., Segall,J.E., Lee,R.J., and Arnold,A. (1999). The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr. Rev. *20*, 501-534.

Polakis,P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. Biochim. Biophys. Acta *1332*, F127-47.

Regnier,C.H., Song,H.Y., Gao,X., Goeddel,D.V., Cao,Z., and Rothe,M. (1997). Identification and characterization of an IkappaB kinase. Cell *90*, 373-383.

Rothwarf,D.M., Zandi,E., Natoli,G., and Karin,M. (1998). IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature *395*, 297-300.

Rudolph,D., Yeh,W.C., Wakeham,A., Rudolph,B., Nallainathan,D., Potter,J., Elia,A.J., and Mak,T.W. (2000). Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. Genes Dev. *14*, 854-862.

Seidensticker,M.J. and Behrens,J. (2000). Biochemical interactions in the wnt pathway. Biochim. Biophys. Acta *1495*, 168-182.

Shtutman,M., Zhurinsky,J., Simcha,I., Albanese,C., D'Amico,M., Pestell,R., and Ben,Z. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. U. S. A. *96*, 5522-5527.

Sommers,C.L., Gelmann,E.P., Kemler,R., Cowin,P., and Byers,S.W. (1994). Alterations in beta-catenin phosphorylation and plakoglobin expression in human breast cancer cell lines. Cancer Res. *54*, 3544-3552.

Spiegelman,V.S., Slaga,T.J., Pagano,M., Minamoto,T., Ronai,Z., and Fuchs,S.Y. (2000). Wnt/Beta-catenin signaling induces the expression and activity of Beta-TrCP ubiquitin ligase receptor. Molecular Cell *5*, 877-882.

Takeda,K., Takeuchi,O., Tsujimura,T., Itami,S., Adachi,O., Kawai,T., Sanjo,H., Yoshikawa,K., Terada,N., and Akira,S. (1999). Limb and skin abnormalities in mice lacking IKKalpha. Science *284*, 313-316.

Tetsu O,M.F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-426.

van de Wetering,M., Cavallo,R., Dooijes,D., van Beest,M., van Es,J., Louriero,J., Ypma,A., Hursh,D., Jones,T., Bejsovec,A., Peifer,M., Mortin,M., and Clevers,H. (1997). Armadillo co-activates transcription driven by the product of the drosophila segment polarity gene dTCF. Cell *88*, 789-799.

Winston,J.T., Strack,P., Beer-Romero,P., Chu,C.Y., Elledge,S.J., and Harper,J.W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. Genes Dev. *13*, 270-283.

Woronicz,J.D., Gao,X., Cao,Z., Rothe,M., and Goeddel,D.V. (1997). IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. Science *278*, 866-869.

Yamaoka,S., Courtois,G., Bessia,C., Whiteside,S.T., Weil,R., Agou,F., Kirk,H.E., Kay,R.J., and Israel,A. (1998). Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell *93*, 1231-1240.

Zhu,A.J. and Watt,F.M. (1999). beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development *126*, 2285-2298.

Xu,L., Corcoran,R.B., Welsh,J.W., Pennica,D., and Levine,A.J. (2000). WISP-1 is a Wnt-1 and beta-catenin responsive oncogene. Genes Dev. *14*, 585-595.

Figure Legends

Figure 1: IKK regulates β-catenin signaling and protein levels.

A luciferase reporter containing Tcf/LEF response elements (TopFlash) was used to detect the effects of IKK on β-catenin signaling. All transfections were normalized with equal amounts of DNA and performed in triplicate. Data is measured in lumens and plotted as percent control. A) SW480 cells were transfected using lipofectamine plus with PCDNA3-cat control DNA and/or 0.1 μg constitutively active (CA) IKK α (S176/180E) or IKK β (S177/81) as well as 0.1 μg of TopFlash or a NF-κB luciferase promoter and 0.002 μg of Renilla luciferase, which was transfected as a control for transfection efficiency. Both CA IKKα and CA IKK β decreased β-catenin signaling by 80-90%. CA IKKα increased NF-κB reporter activity 5 fold. B) SW480 cells were transfected with 0.2 μg of kinase dead IKKα (K44M) or IKKβ (K44A), 0.1 μg of TopFlash, and 0.002 μg of Renilla. Kinase dead IKKα and β had no effect on β-catenin signaling indicating that IKK kinase activity is necessary. C) SKBR3 cells were transfected with 1 μg of CA or dominant negative (DN) IKKα (S176/180A) or IKK β (S177/181A) as well as 1 μg of TopFlash, and 0.02 μg Renilla. DN IKKα increased β-catenin signaling by over 5 fold whereas DN IKKβ had no effect. CA IKKα and IKKβ also had no effect on β-catenin signaling. FopFlash, which is a Tcf/LEF mutated luciferase reporter, was used as a control and was unaffected by IKK. D) SKBR3 cells were transfected with 10 μg DN IKKα or β. NP-40 lysates were probed using a monoclonal antibody to β-catenin. DN IKKα significantly increased β-catenin protein levels whereas DN IKKβ had no effect. SKBR3 cells were also co-transfected with 10 μg of wild-type (WT) β-catenin and DN IKKα and probed for β-catenin expression. Overexpression of WT β-catenin generates a phosphorylated form of β-

catenin and two bands (arrows). The phosphorylated form of β-catenin was not present in cells expressing DN IKKα. E) NP-40 lysates of cell lines used in this study were probed using monoclonal antibodies to IKKα and IKKβ. Jurkat cell lysates were used as a control.

Figure 2: DN IKKα increases nuclear β-catenin levels.

Immunocytochemistry was performed to determine the effect of DN IKKα on β-catenin localization in SKBR3 cells. Cells were transfected with 1 μg of HA-tagged DN IKKα for 48 hours, fixed in 2% paraformaldehyde and 0.2% Triton, and stained with monoclonal HA antibody (A1). DN IKKα was found in both the cytoplasm and nucleus in the majority of transfected cells. These cells were also double-stained for endogenous β-catenin using a polyclonal β-catenin antibody (A2). β-catenin levels were significantly higher in transfected cells and in most cases co-localized with DN IKKα in the nucleus. In SKBR3 cells transfected with 1 μg of FLAG-tagged DN IKKβ, DN IKKβ was found predominantly in the cytoplasm (B1). Increased β-catenin protein levels were not observed nor was β-catenin detected in the nucleus. C) Cos-7 cells were transfected with HA-tagged IKKα or Flag-tagged IKKβ. Nuclear extract (NE, lane 1 and 3) and cytoplasmic fractions (S100, lane 2 and 4) were subjected to western blot analysis using anti-HA (lane 1 and 2) and anti-Flag (lane 3 and 4) for IKKα and IKKβ respectively. Substantially more IKKα than IKKβ was found in the nuclear extract.

Figure 3: IKK regulates cyclin D1 promoter activity.

To determine if IKK could regulate the expression of a β-catenin target gene, a cyclin D1-luciferase promoter (0.5 μg) was used. Both CA IKKα and CA IKKβ decreased cyclin D1

promoter activity in SW480 cells. (Note that data is reported as arbitrary units.) A cyclin D1 promoter with mutated Tcf/LEF sites (ΔLEF) was used as a control.

Figure 4: DN IKKα inhibits the ability of APC to decrease β-catenin signaling.

To determine the effects of IKKα on APC activity, SW480 cells were co-transfected with 0.1 μg APC and 0.1 μg DN IKKα or β in addition to TopFlash and Renilla. APC decreases β-catenin TopFlash signaling by 80 %. (Data is reported as percent control.) DN IKKα but not DN IKKβ completely inhibited the ability of APC to decrease β-catenin signaling.

Figure 5: IKKα interacts with β-catenin.

Superdex-200 column fractions derived from S100 Cos-7 cells transfected with Flag-tagged IKKα and Ha-tagged β-catenin were immunoprecipitated with mouse IgG (lane 1 and 3) or anti-Flag (lane 2) or anti-HA antibody (lane 4). The immunoprecipitates were subjected to fractionation by SDS PAGE, and immunoblotted with antibodies to HA (lanes 1 and 2) or Flag (lanes 3 and 4). β-catenin associates with IKKα.

Figure 6: Potential model for IKKα regulation of β-catenin.

Wnt signaling increases β-catenin protein levels by inactivating GSK-3. We have shown that IKKα directly regulates β-catenin protein levels and signaling activity. This suggests that elevation of β-catenin leads to IKKα activation and subsequent β-catenin phosphorylation and degradation completing a feedback loop. It is possible that β-catenin regulates transcription or activation of an IKK kinase, such as NIK, thus activating IKK and down-regulating signaling. We also showed that IKKα activation is necessary for APC regulated inhibition of β-catenin

signaling. APC may be necessary to facilitate β-catenin targeting by IKKα. In addition, βTRCP is upregulated by β-catenin, consequently decreasing β-catenin levels and down-regulating its own activity as well as increasing NF-κB activity. These data indicate a significant amount of "cross-talk" between these two pathways.

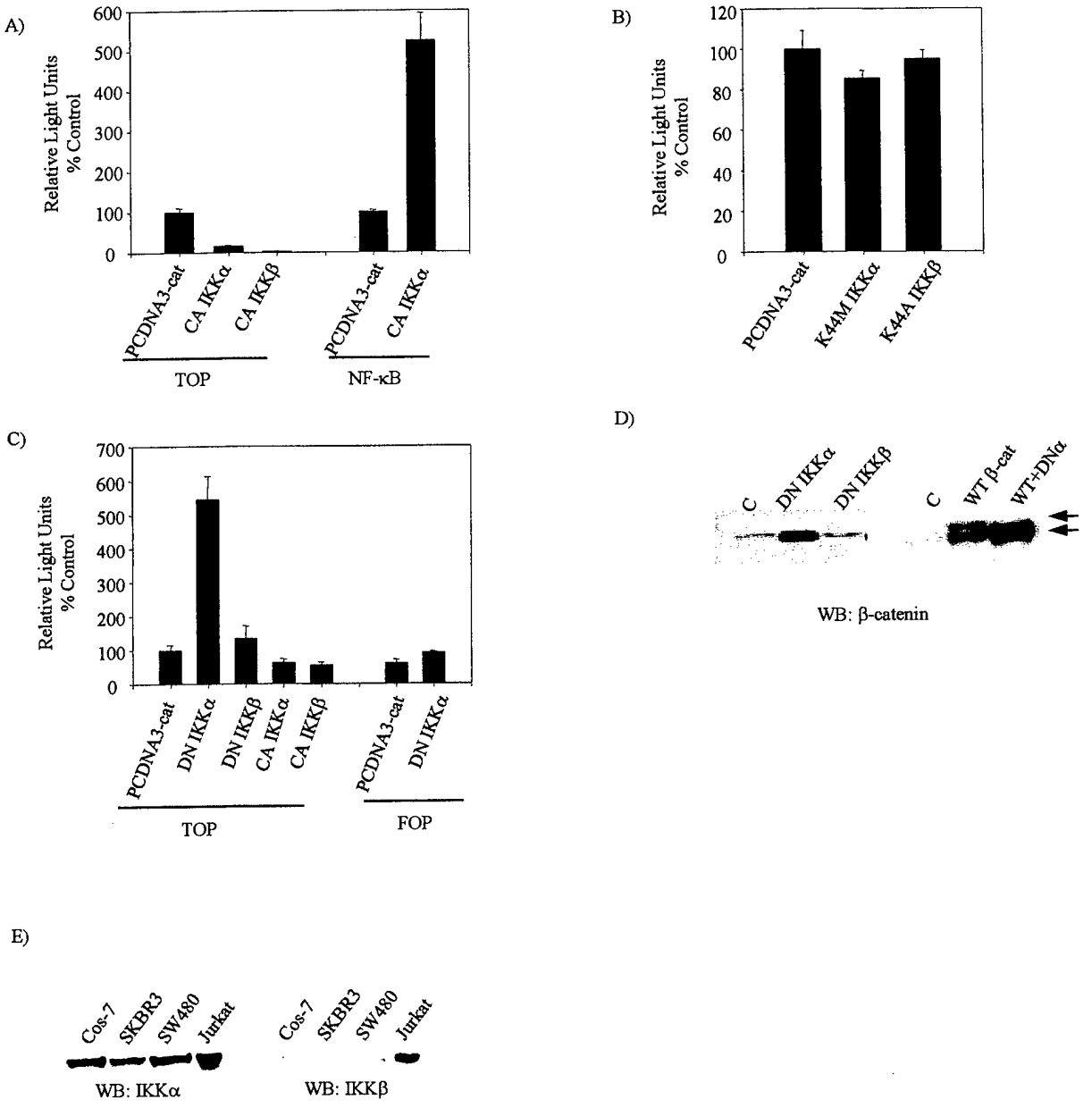
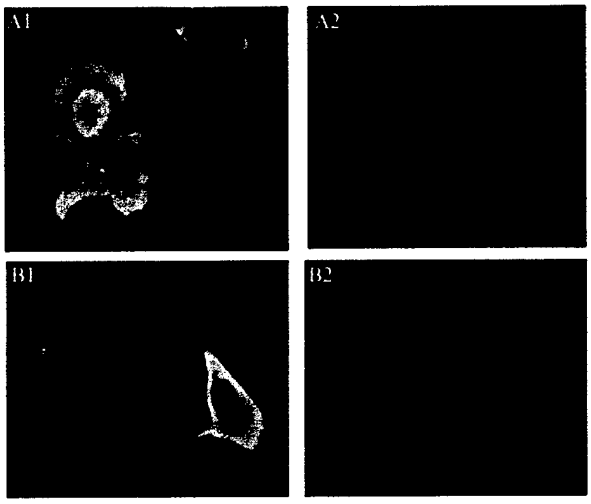

A)
Relative Light Units
% Control
600
500
400
300
200
100
0
PCDNA3-cat
CA IKKα
CA IKKβ
PCDNA3-cat
CA IKKα
TOP
NF-κB
B)
Relative Light Units
% Control
120
100
80
60
40
20
0
PCDNA3-cat
K44M IKKα
K44A IKKβ
C)
Relative Light Units
% Control
700
600
500
400
300
200
100
0
PCDNA3-cat
DN IKKα
DN IKKβ
CA IKKα
CA IKKβ
PCDNA3-cat
DN IKKα
TOP
FOP
D)
C
DN IKKα
DN IKKβ
C
WT β-cat
WT+DNα
WB: β-catenin
E)
Cos-7
SKBR3
SW480
Jurkat
WB: IKKα
Cos-7
SKBR3
SW480
Jurkat
WB: IKKβ

Figure 1



C)

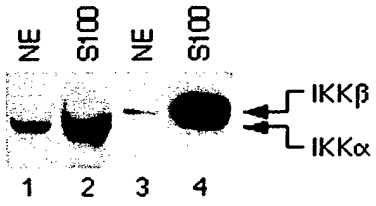


Figure 2

A)

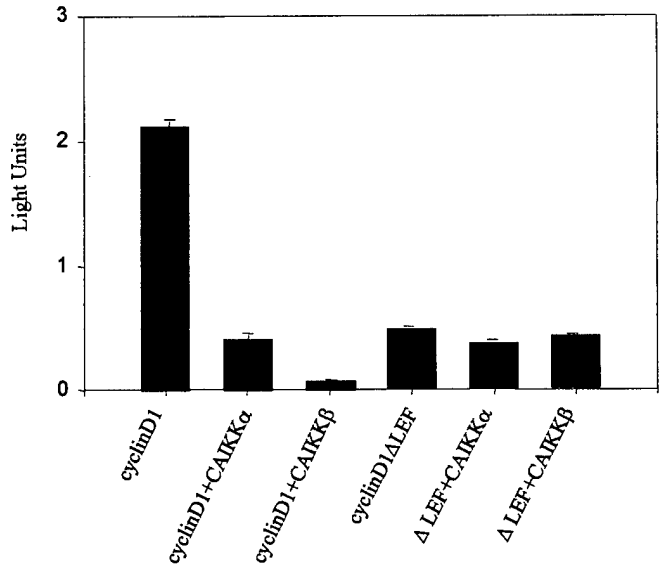

3
2
1
0
Light Units
cyclinD1
cyclinD1+CAIKKα
cyclinD1+CAIKKβ
cyclinD1ΔLEF
Δ LEF+CAIKKα
Δ LEF+CAIKKβ

Figure 3

A)

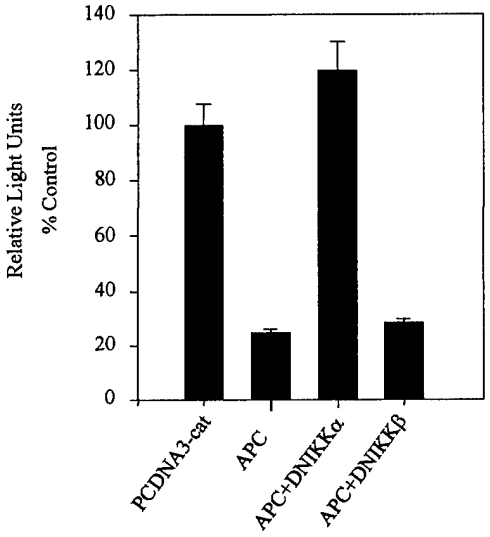

Relative Light Units
% Control
140
120
100
80
60
40
20
0
PCDNA3-cat
APC
APC+DNIKKα
APC+DNIKKβ

Figure 4

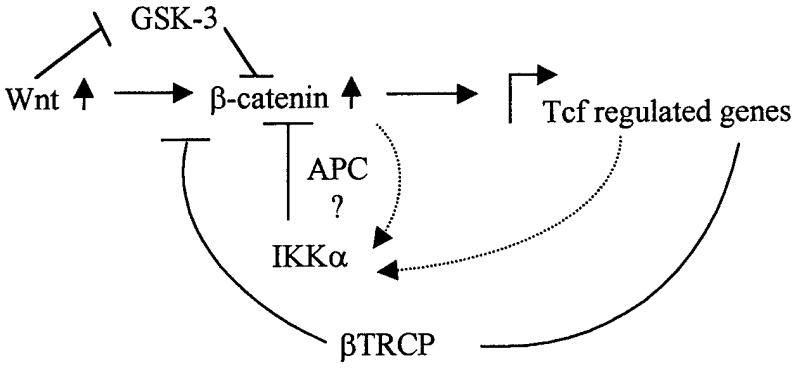


Figure 6